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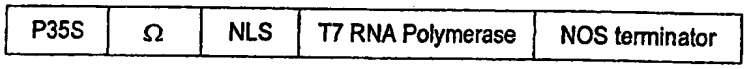

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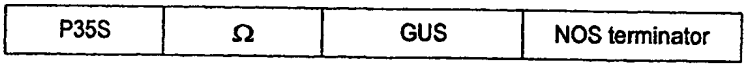

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(57) Abstract

The invention relates to an expression–silencing system comprising a first DNA construct comprising a nucleotide sequence corresponding to the T7 RNA polymerase gene (T7–pol) which carries an NLS sequence, and at least one promoter and at least one terminator sequence operably linked to the T7–pol; a second DNA construct comprising a T7 promoter sequence (pT7), at least one targeting sequence downstream to said pT7 and at least one 3' non–translated terminator sequence operably linked to the targeting sequence; which system can, upon its introduction into a cell, substantially silence the expression at the RNA level of a target sequence in the cell, in a tissue or organ regenerated from said cell, or in a progeny thereof, substantially silenced, by causing the substantial disappearance of the RNA or RNA transcript carrying said sequence or a functional part thereof.

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AN EXPRESSION SILENCING SYSTEM AND DIFFERENT USES THEREOF

FIELD OF THE INVENTION

The present invention relates to an expression-silencing system which is capable, upon introduction thereof into a selected cell, of rendering the expression, at the RNA level, of a target sequence in said cell, in a tissue or organ regenerated therefrom or in a progeny thereof, substantially silenced. The invention also concerns the different uses of the expression-silencing system of the invention.

BACKGROUND OF THE INVENTION

There have been some attempts by scientists to increase expression levels of transgenes in transgenic animals and plants by the introduction of a bacteriophageT7 polymerase/T7 promoter (T7-pol/pT7) system thereinto. T7 RNA polymerase (T7-pol) is a single polypeptide of ~98 kDa which specifically recognized the short viral promoter pT7 [Dunn J.J. *et al.* J. Mol. Biol. **166**:477-535 (1983); Moffatt B.A. *et al.* J. Mol. Biol. **173**:265-269 (1984)]. T7-pol does not require auxiliary proteins for transcription [Chamberlin M., and Ryan T. The Enzymes Ed. Boyer P.D. Academic Press N.Y **15**:87-108 (1982)] and recognizes a single (albeit not stringent) terminator.

It was suggested that any gene placed under the control of the T7 promoter will be specifically and strongly transcribed by a cloned T7 RNA polymerase, which was proven to be successful in bacteria [Tabor S., and Richardson C., Proc. Natl. Acad. Sci USA **82**:1074-1078 (1985); Studier F.W., and Moffatt, B.A., J. Mol. Biol. **189**:113-130 (1986)], and somewhat successful in animal cells [Fuerst T.R., *et al.* Proc. Natl. Acad. Sci. USA **83**:8122-8126 (1986); Fuerest T.R., *et al.* Mol. Cell. Biol. 7:2538-2544 (1987)] Dunn J.J., *et al.* Gene **68**:259-266 (1988); Lieber, A., *et al.* Nucl. Acids Res. **17**:8485-8493 (1989); Moss, B., *et al.* Nature **348**:91-92 (1990)].

The situation in plants is more vague. The expression of T7-pol in tobacco protoplasts and the NLS-direction of the expressed enzyme to the nucleus has been reported [Lassner M.W *et al.* Plant Mol. Biol. **17**:229-234 (1991)]. In addition, the expression of either T7-pol or a

reporter gene driven by the T7-pol/pT7 system in plants was described [Caviedes, M.A., *et al.*, Abstract #479 of the 4th Intl Congress of Plant Mol. Biol (1994); Tuttle A., *et al.* Abstract #478 of the 4th Intl Congress of Plant Mol. Biol (1994); Weschke W., Abstract #480 of the 4th Intl Congress of Plant Mol. Biol (1994)]. A more successful T7-pol/pT7 expression system was described [Tuttle, A. *et al.* Abstracts of the 4th Int. Congress of Plant Mol Biol #478 (1994)] in which T7-pol expressed in tobacco but targeted to the protoplast, directed expression of GUS. A T7-derived system which promotes expression in plants was also described in the course of a novel method to produce hybrid seeds [U.S. Patent No. 5,659,124] and in plastids which may be considered as prokaryotic cells [McBride, K.E. *et al.* Proc. Natl. Acad. Sci. USA **91**:7301-7305 (1994); U.S. Patent 5,545,817].

Gene silencing in transgenic plants is a documented phenomenon, which relates to the introduction of a foreign gene into a cell thereby inducing its silencing, rather than its expression [Cox K.H., and Goldberg R.B. in Plant Molecular Biology, A Practical Approach (Shaw, C.H., ed). Washington DC, IRL Press, pp.1-35 (1988); Baulcombe D.C., and English J.J., Curr. Opin. Biotechnol. **7**:173-180 (1996); Meyer P., and Saedler, H., Annu. Rev. Plant Physiol. Plant Mol. Biol. **47**:23-48 (1996); Stam M., *et al.* Ann. Bot. **79**:3-12 (1997)]. In general, the insertion of a particular gene into a plant may cause the silencing of homologous native or transgenic genes, which is referred to as "co-suppression" [Depicker A., *et al.* Curr. Opin. Cell Biol. **9**:373-382 (1997); Matzke M.A., *et al.* EMBO J. **8**:643-649 (1989); Matzke M.A., *et al.* Mol. Gen. Genet. **238**: 379-386 (1993); Napoli C., *et al.* Plant Cell **2**:279-289 (1990)]. Co-suppression may also be caused by the introduction of homologous RNAs into the cells, such as viral RNAs [Lindbo J.A., *et al.* Plant Cell **5**:1749-1759 (1993); Mueller E., *et al.* Plant J. **7**:1001-1013 (1995)].

Silencing may occur at the transcriptional level, i.e. inhibition of transcription [Flavell R.B. Proc. Natl. Acad. Sci. USA **91**:3490-3496 (1994); Matzke M.A. *et al.* in Homologous Recombination and Gene Silencing in Plants Ed. Paszkowski J. Kluwer Academic Publishers, Dordrecht 271-307] or post-transcriptionally [Van Blokland R. *et al.* Plant J. **6**:861-877 (1994); Boerjan W., *et al.* Plant Cell **6**:1401-1414 (1994); De Carvalho-Niebel F., *et al.* Plant Cell **7**:347-358 (1995)]. It has been suggested that silencing at the

post-transcriptional level is caused by degradation of the primary transcript of the expressed gene, thus no mRNA and no protein will be found in the cytoplasm [Tanzer M.M., *et al.* Plant Cell **9**:1411-1423 (1997)]. Furthermore, silencing-triggering mechanisms involving the sensing of RNA levels [Goodwin J., *et al.* Plant Cell **8**:95-105 (1996); Smith C.J.S., *et al.*, Mol. Gen. Genet. **224**:477-481 (1990); Metzlaff M., *et al.* Cell **88**:845-854 (1997)] or ectopic pairing of homologous DNA sequences [Baulcombe D.C., and English J.J., Curr. Opin. Biotechnol. 7:173-180 (1996); Matzke M.A., Dev. Genet **11**: 214-223 (1990)] have also been suggested. Plant cells which have been silenced for a certain viral transgen and thus became resistant to virus infection due to the specific degradation of viral sequences have also been described [Lindbo J.A., *et al.* (1993) *ibid.*; Goodwin J., *et al.* (1996) *ibid.*].

SUMMARY OF THE INVENTION

The present invention relates to an expression-silencing system comprising:- (a) a first DNA construct comprising a nucleotide sequence corresponding to the T7 RNA polymerase gene (T7-pol) or to a functional equivalent or fragment thereof, which sequence carries an NLS sequence, the construct further comprising at least one promoter and at least one terminator sequence operably linked to said T7-pol; (b) a second DNA construct comprising a T7 promoter sequence (pT7) or a functional fragment thereof, at least one targeting sequence downstream to said pT7 and at least one 3` non-translated terminator sequence operably linked to said targeting sequence; which system is capable, upon introduction thereof into a cell, of rendering the expression at the RNA level of a target sequence in said cell, in a tissue or organ regenerated from said cell, or in a progeny thereof, substantially silenced, by causing the substantial disappearance of the RNA or RNA transcript carrying said sequence or a functional part thereof.

In the same aspect, the invention relates to an expression-silencing system comprising a nucleotide sequence corresponding to the T7 RNA polymerase gene (T7-pol) or a functional equivalent or fragment thereof which sequence carries an NLS sequence, the construct further comprising at least one promoter and at least one terminator sequence operably linked to said T7-pol, a T7 promoter (pT7) or a functional equivalent or fragment thereof, at least one targeting sequence downstream to said the pT7, and at least one

additional terminator sequence operably linked to said nucleic acid sequence of interest, said system being capable, upon introduction thereof into a cell, of rendering the expression at the RNA level of a target sequence in said cell, in a tissue or organ regenerated from said cell, or in a progeny thereof, substantially silenced, by causing the substantial disappearance of the RNA or RNA transcript carrying said sequence.

In a second aspect, the invention relates to a process for the transformation of a plant with a gene-silencing system, which process comprises:- (a) transforming plant cells with the expression-silencing system of the invention; (b) selecting the plant cells transformed with at least one DNA construct according to (a) and regenerating said selected cells to provide a differentiated flowering plant; and (c) hybridizing a plant transformed with said first DNA construct with a plant transformed with said second DNA construct, which first plant and second plant are obtained in (b), said hybridization thus providing a double-transformed plant in which the expression of a target sequence is substantially suppressed.

Further, the invention relates to a method for producing a transgenic plant carrying a substantially silent target sequence, by hybridizing a plant carrying and expressing said target sequence with a transformed plant obtained by the process of the invention.

Still further, the invention relates to a method for producing a transgenic plant carrying a substantially silent target sequence, by grafting a plant, or parts thereof, carrying and expressing said silent target sequence on a transformed plant obtained by the process of the invention.

Yet further, the invention relates to a method of silencing the expression of a target sequence within the genome of a plant or within the genome of a plant-infecting pathogen, which method comprises the steps of: (a) providing a first plant capable of regenerating; (b) hybridizing said first plant with a second plant transformed with the expression-silencing system of the invention; and (c) selecting those plants obtained by the hybridization of step (b), in which the expression of said target sequence is substantially silenced.

Finally, the invention relates to a method of identifying a nucleic acid of interest within the genome of a plant, wherein the nucleic acid of interest encodes a pre-defined plant

phenotype, which process comprises the steps of:- (a) providing a first plant carrying in its genome said nucleic acid of interest; (b) transforming said first plant or cells thereof with a second plant or cells thereof, respectively, transformed with the expression-silencing system of the invention; (c) selecting from the population obtained in step (a) transformed plants/plant cells in which the pre-defined phenotype is substantially silenced; and (d) employing said random nucleic acid sequence within the genome of transformed plants selected in step (c) as a probe in screening genomic DNA and cDNA libraries of said first plant, thereby identifying the gene comprising said random nucleic acid sequence, which gene is responsible for said pre-defined phenotype.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1A-1C *Schematic representation of the various constructs introduced into plants.*

Fig. 1A represents the p35S-GUS construct, Fig. 1B represents the p35S-T7pol construct and Fig.1C pT7-GUS construct.

Figure 2 *Western blot analysis for the expression of T7 RNA polymerase.*

This figure represents the Western blot analysis for the expression of T7-pol. Lane 1 indicates protein extract from a non-transformed plant. Lanes 2 to 7 indicate extracts from various double-transformed plants. Lane 8 represents a commercial T7 RNA polymerase.

Figure 3A-3B *Nuclear run-on transcription assay of p35S-GUS and p35S-T7pol/pT7-GUS plants.*

Fig. 3A- Transcripts from p35S-GUS nuclei served as a probe. Fig. 3B- Transcripts from p35S-T7pol/pT7-GUS nuclei served as probes. The various membrane "slots" contain plasmids carrying portions of the following cDNA sequences: GUS, the nuclear subunit of rubisco (Rub), actin (Act), NPTII (Km), T7-pol (T7 RNAP), ubiquitin (Ubiq) and the irrelevant plasmid Bluescript (B.S.). Empty slots are indicated by (-).

Figure 4A-4B *RNase protection assay.*

Fig. 4A shows RNase protection assay with a T7-pol probe, presented by the two left lanes and RNase protection with a GUS probe presented by the two right lanes. Fig. 4B shows the RNase protection assay with an actin probe. 35S-GUS designates RNA extracted from p35S-GUS-carrying plants, whereas, T7-GUS designates RNA extracted from plants carrying p35S-T7-pol/pT7-GUS. The arrows indicate the expected positions of GUS, T7-pol and actin.

DETAILED DESCRIPTION OF THE INVENTION

In an attempt to construct a "super-expressing" transgenic plant, by transforming tobacco plants with the T7pol/pT7 system, known to increase expression levels in bacteria and, to some extent, in animal cells, the inventors have surprisingly found that plants transformed with the enzyme T7-pol under the control of the constitutive promoter CaMV-35S (p35S), as well as with a reporter gene (GUS) placed under the control of the pT7, were silenced in respect of GUS expression although sufficiently expressing T7-pol.

Thus, the present invention relates to an expression-silencing system comprising: a first DNA construct comprising a nucleotide sequence corresponding to the T7 RNA polymerase gene (T7-pol) or to a functional equivalent or fragment thereof which sequence carries an NLS sequence, the first construct further comprising at least one promoter and at least one terminator sequence operably linked to the T7-pol; and a second DNA construct comprising a T7 promoter sequence (pT7) or a functional fragment thereof, at least one targeting sequence downstream to said pT7 and at least one 3` non-translated terminator sequence operably linked to the targeting sequence; which system is capable, upon introduction thereof into a cell, of rendering the expression at the RNA level of a target sequence in said cell, in a tissue or organ regenerated from said cell or progenies thereof, substantially silenced, which silencing is caused by the substantial disappearance of the RNA or RNA transcript carrying said sequence or a part thereof.

Alternatively, the expression-silencing system of the invention may comprise a nucleotide sequence corresponding to the T7 RNA polymerase gene (T7-pol) or a functional equivalent or fragment thereof which sequence carries an NLS sequence, the system further comprising at least one promoter and at least one terminator sequence operably linked to said T7-pol, a T7 promoter (pT7) or a functional equivalent or fragment thereof, at least one targeting sequence downstream to the pT7, and at least one additional terminator sequence operably linked to said nucleic acid sequence of interest, said system being capable, upon introduction thereof into a cell, of rendering the expression at the RNA level of a target sequence in said cell, in a tissue or organ regenerated from said cell, or in progenies thereof, substantially silenced, by causing the substantial disappearance of the RNA or of an RNA transcript carrying said sequence.

By the term 'functional equivalent/analogue/fragment thereof' is meant any variant of the nucleic acid sequence which retains the biological function of the peptide, protein or protein product transcribed therefrom or any variant of the protein or peptide itself which retain their biological function. Such variants may include modifications, mutations, deletions replacements and/or insertions within the naturally occurring sequence.

The term 'targeting sequence' for purposes defined herein refers to any exogenous sequence which is introduced into a selected cell by means known to the man of the art, such as by employing the expression-silencing system defined herein. Exogenous nucleic acid sequences according to the invention can possess sequences identical or substantially homologous to an endogenous sequence/s or to a part thereof which endogenous sequence/s are present in the cell prior to introduction of said system into said cell. Yet, the exogenous nucleic acid sequence can possess sequences identical or substantially homologous to a pathogenic nucleic acid sequence present in said cell through infection thereof by a pathogen. Naturally, the expression-silencing system of the invention may contain one or more targeting sequences.

The targeting sequences according to the invention are those which permit integration into the genome of the selected cell containing the target gene of interest or into any other nucleic acid sequence present in said cell either prior to introduction of the system of the invention into the cell or as a result of a later infection. Such sequences include those encoding a pathogenic product (e.g. a protein or a peptide), irrespective of whether the pathogenic genome will constitute an integral part of the genome of the cells.

The targeting sequences may be a coding or a non-coding nucleic acid sequence either lying upstream of the transcriptional start site, within the primary transcript, or downstream of the transcriptional stop site of the nucleic acid of interest (target sequence), or the targeting sequence may be any sequence present in the cell through a previous modification. Thus, the targeting sequence or sequences according to the invention may, independently, correspond to the sequence within a gene of interest (such as, the sequences of an exon and/or intron), immediately adjacent to a gene of interest (i.e., with no additional nucleotides between the targeting sequence and the coding region of the gene of interest), upstream gene of interest (such as the sequences of the upstream non-coding

region or promoter sequences), or upstream of and at a distance from the gene (such as, sequences upstream of the promoter sequences).

By the term 'target sequence' is meant any sequence, the suppression of expression of which is desired. The sequence may be endogenous or it may be a sequence of an infecting pathogen. Preferably, the selected cell in which the expression at the RNA level is substantially silenced is any eukaryotic or prokaryotic cell such as a plant cell, a mammalian cell, bacteria, yeast, their pathogens or any suitable tissue culture cells.

By the term 'regenerated tissue or organ' is meant any differentiated tissue or organ regenerated from the cell into which the system of the invention was introduced. One example for a regenerated organ according to the invention is a differentiated flowering plant regenerated from a plant cell into which the silencing system of the invention was introduced.

The silencing of expression by the system of the invention occurs at the RNA level as indicated by the disappearance of the RNA carrying the target sequence, a part thereof or a sequence corresponding thereto. A specific example for such disappearance may be seen in Figs. 3 and 4, which show that although the GUS gene is transcribed, no GUSmRNA is detected. The RNA according to the invention can be any RNA sequence, either a coding or a non-coding one or it may be a transcript of an RNA or DNA coding or non-coding sequences.

In particular, the target sequence according to the invention corresponds to:- (a) a gene encoding a protein or a peptide product, the silencing of which is desired; (b) a non-coding nucleic acid sequence, which, under normal conditions, promotes the expression of an essential coding sequence; (c) a nucleic acid sequence which corresponds to (a) or to (b) or to a fragment thereof, within the scope of degeneracy of the genetic code; or (d) a nucleic acid sequence which hybridizes with the sequence according to (a), to (b), or to (c) or with fragments thereof, which hybridization is carried out under conditions which allow such hybridization to occur. The conditions for hybridization vary and include, for example, hybridization at about 50°C in a solution containing 0.9 M of a suitable salt, such as NaCl.

In case the selected cell is a plant cell, the target gene may encode an expressible plant protein or peptide or an expressible protein or peptide product of a plant pathogen.

The protein or peptide product of the plant pathogen may be selected from a plant virus, a bacterium or a fungus, all of which are capable of infecting the plant. One example for a target gene encoding a bacterial protein is the GUS gene that may be introduced into to the plant cell by methods known to the man of the art and is then expressed in the plant cell.

Alternatively, the target gene according to the invention may be a gene that encodes a human protein or peptide product or a protein or peptide product of a human pathogen. Accordingly, the system of the invention will render the expression of the human target gene substantially silenced.

Yet further, the target sequence according to the invention may be a non-coding sequence including, *inter alia*, one or more regulatory elements which, under normal conditions, promotes the expression of a specific coding sequence.

Within the expression-silencing system of the invention, the pT7 corresponds to the promoter region of the bacteriophage T7 or to functional analogues thereof, which promoter is capable of initiating transcription of at least one targeting sequence downstream thereto.

According to one particular embodiment of the invention, the target sequence is the TMV non-coding sequence Ω [Gallie D.R. *et al.* Nucl. Acids Res. **15**:3257-3272 (1987)], the NLS sequence is preferably the SV-40 NLS sequence, the promoter sequence is preferably the plant promoter p35S and the terminator is preferably the NOS terminator. An example for a system consisting of the above elements may be seen in Figure 1. As an alternative to the NOS terminator, the β-1,3-gluconase terminator may be utilized or any other suitable terminator which is capable of terminating the transcription of a nucleic acid sequence and of the adding polyadenylated ribonucleotides to the 3 end of the primary transcript of the target sequence.

Within one embodiment of the system of the invention, the T7 terminator and the NOS terminator are operably linked within the system of the invention to the targeting sequence.

Evidently, any functional equivalent or fragment of the NLS sequence, the promoter or the terminator sequence may be employed in the expression-silencing system of the invention.

As indicated above, a particular embodiment of the first and second DNA constructs of the invention is shown in Figures 1A and 1B, respectively. As may be understood from the figures, the different constituents comprising each construct are linked in a predefined order to enable the efficient silencing of the target gene in the selected cell, upon introduction of the system thereto.

According to a second aspect, the invention relates to a process for the transformation of a plant with a gene-silencing system which process comprises the steps of:- (a) transforming plant cells with:- (i) a first DNA construct comprising a nucleotide sequence corresponding to the T7 RNA polymerase gene (T7-pol) or a functional equivalent or fragment thereof, at least one plant promoter and at least one plant terminator sequence operably linked to said T7-pol; and with (ii) a second DNA construct comprising a T7 promoter sequence or a functional fragment thereof, a targeting sequence downstream to the T7 promoter, and at least one 3` non-translated terminator sequence operably linked to the targeting sequence, said construct optionally further comprising other additional regulatory elements operably linked to the targeting sequence. The process further comprises the steps of (b) selecting the plant cells transformed with at least one DNA construct according to (a) and regenerating said selected cells to provide a differentiated flowering plant; and (c) hybridizing a plant transformed with said first DNA construct with a plant transformed with said second DNA construct, which first plant and second plant are obtained in (b), the hybridization thus provides a double-transformed plant wherein the expression of a target sequence is substantially suppressed. Evidently, cells transformed with both constructs at a single process stage may be utilized for further hybridizations as described herein after.

Within the same aspect, the invention also concerns to a process for the transformation of plant with a gene-silencing system, which process comprises the steps of:- (a) transforming plant cells with a DNA construct comprising a nucleotide sequence corresponding to the T7 RNA polymerase gene (T7-pol) or a functional equivalent or fragment thereof which sequence carries an NLS sequence, the construct further comprising at least one plant promoter sequence and at least one plant terminator sequence operably linked to the polymerase gene, a T7 promoter sequence (pT7) or a functional fragment thereof, a targeting sequence downstream to said pT7, and at least one additional terminator

sequence operably linked to said targeting sequence, which DNA construct is capable, upon transformation thereof into a plant, of rendering the expression of a target sequence in said plant or in its progeny, substantially silenced; (b) selecting plant cells transformed with said DNA construct according to (a) and regenerating said selected cells to provide such differentiated flowering plant.

According to this process the targeting sequence substantially corresponds to the target sequence or to a fragment thereof. In addition, the target sequence according to the process disclosed herein corresponds to:- (a) a gene encoding a protein or a peptide product, the silencing of which is desired; (b) a non-coding nucleic acid sequence, which, under normal conditions, promote the expression of an essential coding sequence; (c) a nucleic acid sequence which corresponds to (a) or to (b) or to a fragment thereof, within the scope of degeneracy of the genetic code; or (d) a nucleic acid sequence which hybridizes with the sequence according to (a), to (b), or to (c) or with fragments thereof, which hybridization is carried out under conditions which allow such hybridization to occur. Such hybridization conditions vary and include, *inter alia*, hybridization at 50°C in a suitable electrolyte, such as NaCl, solution (0.9M).

As may be seen in the following Examples, upon introduction of the expression-silencing system of the invention (Figs. 1A and 1B), containing as the target gene the sequence encoding the GUS protein, into a plant cell that was previously engineered to express this bacterial protein, the expression of the gene was suppressed.

In a third aspect, the invention relates to a method for producing a transgenic plant carrying a substantially silent target sequence, by hybridizing a plant carrying and expressing said target sequence with a transformed plant obtained by the process of the invention.

In yet another aspect, the invention relates to a method for producing a transgenic plant carrying a substantially silent target sequence, by grafting a plant, or parts thereof, carrying and expressing said silent target sequence on a transformed plant obtained by the process of the invention, as shown in Example 4.

Transgenic plants or progenies thereof obtained by the above disclosed methods, in which the expression of a predefined target sequence is substantially suppressed as the result of said hybridization, are also within the scope of the invention.

In a further aspect, the invention concerns with a method of silencing the expression of a target sequence within the genome of a plant or within the genome of a plant infecting pathogen present in the cell prior to the following manipulations, which method comprises the steps of:- (a) providing a first plant capable of regenerating; (b) hybridizing said first plant with a second plant transformed with:- (i) a first DNA construct comprising a nucleotide sequence corresponding to the T7 RNA polymerase gene (T7-pol) or a functional equivalent or fragment thereof which sequence carries an NLS sequence, said construct further comprising at least one plant promoter and at least one plant terminator sequence operably lined to said sequence; and with (ii) a second DNA construct comprising a T7 promoter sequence (pT7), a targeting sequence downstream to said pT7 and a 3` non-translated terminator sequence operably linked to said targeting sequence, said construct optionally further comprising additional regulatory elements operably linked to said targeting sequence, said plant being referred to as a double-transformed plant; and (c) selecting plants obtained by the hybridization of step (b), in which the expression of said target sequence is substantially silenced.

Alternatively, the method of silencing the expression of a target sequence within the genome of a plant according to the invention or with the genome of a plant infecting pathogen present in the cell prior to the following manipulations may comprise the steps of:- (a) providing a first plant comprising said target sequence, which plant is capable of regenerating; (b) hybridizing said first plant with a second plant transformed with a DNA construct comprising a nucleotide sequence corresponding to the T7 RNA polymerase gene (T7-pol) or a functional equivalent or fragment thereof which sequence carries an NLS sequence, said construct further comprising a plant promoter and a plant terminator sequence operably linked to said T7-pol, a T7 promoter (pT7) or a functional fragment thereof, a targeting sequence downstream to said pT7, and at least one additional promoter sequence operably linked to said targeting sequence and; (c) selecting plants obtained by the hybridization of step (b), wherein the expression of said target sequence is substantially silenced.

In any case, the targeting sequence within the system will substantially corresponds to the target sequence or to a fragment thereof and the target sequence may correspond to:- (a) a gene encoding a protein or a peptide product, the silencing of which is desired; (b) a non-coding nucleic acid sequence, which, under normal conditions, promotes the expression of an essential coding sequence; (c) a nucleic acid sequence which corresponds to (a) or to (b) or to a fragment thereof, within the scope of degeneracy of the genetic code; or (d) a nucleic acid sequence which hybridizes with the sequence according to (a), to (b), or to (c) or with fragments thereof, which hybridization is carried out under conditions which allow such hybridization to occur.

Finally, the invention concerns with a method of identifying a nucleic acid of interest within a plant's genome wherein the nucleic acid of interest encodes a pre-defined plant phenotype, which process comprises the steps of:- (a) providing a first plant comprising within its genome said nucleic acid of interest; (b) transforming said first plant with a second plant transformed with:- (i) a first DNA construct comprising a nucleotide sequence corresponding to the T7 RNA polymerase gene (T7-pol) or a functional equivalent or fragment thereof which sequence carries an NLS sequence, said construct further comprising at least one plant promoter and at least one plant terminator sequence operably lined to said sequence; and with (ii) a second DNA construct comprising a T7 promoter sequence, a random nucleic acid sequence downstream to said T7 promoter, and a 3` non-translated terminator sequence operably linked to said random nucleic acid sequence, said construct optionally further comprising additional regulatory elements operably linked to said nucleic acid of interest, said transformation thus provides a population of transgenic plants; (b) selecting from the population obtained in step (a) transformed plants/plant cells in which the pre-defined phenotype is substantially silenced; and (c) employing said random nucleic acid sequence within the genome of the transformed plants selected in step (c) as a probe in screening genomic DNA and cDNA libraries of said first plant, thereby identifying the gene comprising said random nucleic acid sequence which gene is responsible for said pre-defined phenotype.

Alternatively. the method of identifying a nucleic acid of interest within a plant's genome wherein said nucleic acid of interest encodes a pre-defined plant phenotype, may comprise the steps of:- (a) providing a first plant comprising within its genome said nucleic acid of

interest; (b) transforming said first plant or with a second plant transformed with a DNA construct comprising a nucleotide sequence corresponding to the T7 RNA polymerase gene (T7-pol) or a functional equivalent or fragment thereof which sequence carries an NLS sequence, said construct further comprising at least one plant promoter sequence and at least one plant terminator sequence operably linked to said T7-pol, said DNA construct further comprising a T7 promoter sequence or a functional fragment thereof, a random nucleic acid sequence downstream to said T7 promoter, and a 3` non-translated terminator sequence operably linked to said random nucleic acid sequence; and (c) selecting from the plants obtained in step (b) those transformed plants in which the pre-defined phenotype is substantially silenced; and (d) employing said random nucleic acid sequence within the genome of the transformed plants selected in step (c) as a probe in screening genomic DNA or cDNA libraries of said first plant, thereby identifying the gene comprising said random nucleic acid sequence, which gene is responsible for said pre-defined phenotype.

Evidently, any further uses of the expression-silencing system, the process for the transformation of a selected cell with said system, the transgenic plant introduced with the system of the invention and the different methods of the invention, are also within the scope of the invention.

The invention will now be described in an illustrative manner and it is to be understood that the terminology which will be used is intended to be in the nature of the words of description rather than of limitation.

Obviously, many modifications and variations of the present invention are possible in light of the above teaching. It is therefore, to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described.

EXAMPLES

Example 1 - General methodology

DNA sequences were isolated from plants, from bacteriophage T7 and from the virus tobacco mosaic virus (TMV) and propagated in a suitable plasmid (e.g. pBluescript, pCR2.1) in *E.coli* JM109 by a standard procedure [Sambrook J., *et al.* Molecular Cloning, A Laboratory Manual. Cold Spring Harbor Press (1989); Ausbubel F.M., *et al.* Current

Protocols in Molecular Biology. John Weily & Sons Inc. (1995)]. Alternatively, DNA sequences to be transferred to plants were provided by the binary plasmids (such as the pBI101). The DNA sequences utilized were the plant promoter p35S, the bacteriophage gene for T7 RNA polymerase (T7-pol), the plant terminator NOS, the bacteriophage terminator (T7-terminator), the translation enhancer from TMV (Ω) and the coding sequence of the bacterial gene for β-glucuronidase (GUS).

Cloning of T7-pol and GUS

The gene for T7-pol was 5`-fused with the Ω-translation-enhancer element from TMV and the SV-40 element, NLS, directing protein transport to the nucleus. This construct was placed between a CaMV-35S promoter (p35S) and a NOS terminator and was designated-35S-T7-pol. Two constructs of GUS were prepared, both 5`-fused to Ω: one construct p35S-GUS was placed between p35S and the NOS terminator, and the other (pT7-GUS) between pT7 and, assuming that T7-pol may not recognize plant termination signals, two terminators: the plant NOS terminator and the bacteriophage T7 terminator. All constructs also carried the NPTII gene for Km resistance, and were transferred into the binary plasmids pGA643 and pBI101 [Vardi E., *et al.* Proc. Natl. Acad. Sci. USA **90**: 7513-7517 (1993)], which provided border sequences for integration into the plant's genome and cassettes for a selectable marker in plants (kanamycin resistance). Figure 1A and 1B show the constructs obtained. Figure 1C represents a positive control wherein the cassette introduced into the binary plasmid pBI101 carries the GUS coding sequences between p35S and the NOS terminator.

GUS assay

Gus activity was assayed either histochemically or by fluorometric determination of the production of 4-methylumbelliferone (4-MU) from its substrate 4-methylumbellferyl glucuronide (MUG, sigma) according to the methodology described [Jefferson R.A., Plant Mol. Biol. Rep. 5:387-405 (1987)].

RNA assay

All assays were carried out with p35S-T7-pol/pT7-GUS plants and with 35S-GUS plants as controls. Since the absence of GUS expression could have been attributed to the

expression of an inactive T7-pol, or to its inability to move to the nucleus and affect transcription, initial transcription was tested first by nuclear run-on assays. As indicated in Figure 3, GUS transcripts were driven from p35S as well as from pT7. The level of GUS transcription from pT7 was comparable to that of the expressed T7-pol and NPTII genes (driven by p35S and pNOS, respectively), but higher than the internal control of the ubiquitin gene under its own native promoter. As expected, the control p35S-GUS plants readily transcribed GUS.

RNase-protection assays, however, indicated that while T7-pol-mRNA and actin-mRNA are present in the 35S-T7-pol/pT7-GUS plants, GUS-mRNA is absent. GUS-mRNA and the internal control actin-mRNA were both detected in the control p35S-GUS plants (Fig. 4). Silencing of GUS expression in a plant T7-pol/pT7 system therefore occurs at a post-transcriptional stage.

Nuclear run-on assay

The procedure was carried out essentially as described by Cox and Goldberg [Cox K.H., and Glodberg R.B, In Plant Molecular Biology, A Practical Approach (Shaw C.H. Ed) Washington DC IRL Press pp 1-35]. Reduction of the starch content was found to be essential for a good preparation. Therefore, plants were stored in the dark for 48 hr prior to nucleic isolation. Nuclei were finally separated on 40-76% percol gradients and stored as 100 µl aliquots at -80°C.

In order to enable quantitative comparisons, relevant plasmids (1 µg per 1000 bp) were boiled and quickly cooled, and the denatured plasmid was slot-blotted onto a nitrocellulose membrane. A regular Southern-type procedure was then performed with the nuclear RNA preparation as a probe.

RNase protection assay

GUS mRNA electrophoresed to about the same position as the 18S rRNA. To avoid background problems, RNase protection was therefore preferred to Northern-blot hybridization.

RNase protection assays were carried out essentially according to the manufacturer's protocol (Ribonuclease Protection Kit Ambion). Three probes were prepared for the RNase protection assays: An internal control actin probe (474 bases) was made by PCR amplification of a segment of the actin cDNA between bases 4282-4756 from tobacco DNA (GenBank #X63603). The amplified product was cloned into the plasmid Bluescript KS (Stratagene). The antisense probe was transcribed from the T3 promoter of the linearized recombinant plasmid (BamH1). The GUS probe was prepared by cleaving our an XbaI-EcoR1 fragment from a GUS carrying plasmid (pBI221, Clonetech) and subcloning it into the plasmid Bluescript. A 760bp antisense probe was prepared by transcribing the MluI-linearized plasmid from the T3 promoter. The probe for T7-pol was a BamH1-EcoR1 fragments subcloned into Bluescript from Tabor and Richardson's plasmid pGP1-2. The antisense probe (240bp) was transcribed from the T7 promoter of the NdeI-linearized plasmid.

Example 2 - Plant transformation and silencing of GUS expression

Agrobacterium tumefaciens carrying a compatible disarmed Ti plasmid (e.g. pEHA101) was transformed with one of the engineered binary plasmids by triparental mating [An G., Methods Enzymol. **153**:292-305 (1987)], or by electroporation [Li-Chin-Ho *et al.* Plant Physiology and Biochemistry **35**:959-968 (1997)] with GenePulser II (performed according to the manufacturer's instructions, Biorad).

Agrobarterium-mediated transformation was carried out, and homozygous plants were selected at the R2 stage as described previously [Vardi E., *et al.* (1993) *ibid.*]. In particular, tobacco and tomato leaf discs were inoculated with the transformed *A. tumefaciens* according to standard procedure [An G. (1987) *ibid.*]. Agro-inoculated leaf discs were placed on the following medium: MS salts (4.71 gr/L) [Murashige T. and Skoog F. Physiol. Plant. **15**:485-497 (1962)], sucrose (20 gr/L), maniol (10 gr/L), Nobel Agar (10 gr/L), seatin (2 mg/L), IAA (indole acetic acid 0.1 Gr/L), pH 5.8. After 48 hrs in this

medium, the leaf discs were transferred into a fresh medium further containing kanamycin (300μg/ml) and carbenicillin (300μg/ml) which was replaced every 10 days. Callus developed from the leaf discs (20-30 days from Agro-inoculation) was transferred to the same initial medium however containing 400 μg/ml kanamycin. Shoots developing from the callus were transferred individually for root development to a medium containing: MS salts (4.71 g/L), sucrose (30 gr/L), Nobel Agar (10 gr/L). Rooted shoots were then potted and transferred to the greenhouse to grow into mature flowering plants.

As an alternative procedure, plants may be introduced with the relevant vectors also by biolistic methods [Bionard protocols] by bombarding beads coated with the pertinent DNA into plant tissues.

As an alternative to the selection of the transformed plants using a selection marker, efficient selection may be obtained based on PCR identification of the transgene or by histochemical assays for GUS expression, as an alternative or in addition to the selectable marker.

Plants transformed with the construct shown in Fig 1A, which were capable of expressing (transcription and translation) GUS, were hybridized with plants transformed with the cassette comprising pT7, Ω, GUS and two terminators (Fig. 1B). The resultant double-transformed plant expressed T7-pol (Fig 2), transcribed GUS (Fig. 3) but did not produce GUS mRNA (Fig. 4) and thus was unable to express GUS protein (Table 2), which exhibits the silencing of the expression of GUS at the post-transcriptional level. Importantly, the expression of GUS was silenced in all the resulting transgenic plants.

Example 3 - Preparation of progeny silenced plants via pollination (silencing in trans)

Transgenic R0 tobacco plants carrying the p35S-RNA-pol construct were crossed with similar plants carrying the pT7-GUS construct (see Fig. 1 for a diagram of the constructs). The hybrid plants were self-pollinated and progeny were selected for several generations. At all stages plants were PCR-analyzed for both T7-pol and GUS, and only plants carrying both genes were selected. Then, a double transformed 35S-T7-pol/pT7-GUS plant was pollinated by an expressing 35S-GUS control plant. Three of the progeny plants out of 18 were found to carry all three genes (35S-T7-pol, pT7-GUS and 35S-GUS) as determined by PCR using a promoter-specific and a gene-specific primers for each case. None of these

triple-transformed plants exhibited GUS activity either by histochemical staining (data not shown) or by measuring GUS activity in plant's extracts (Table 1). Hence, once the T7-derived silencing machinery was set in motion it was capable of silencing an already active pertinent gene. The evoked silencing mechanism was therefore not a peculiarity of the foreign T7 system but carried relevance to silencing of the gene under a "native" plant promoter.

Many of the 35S-T7-pol/pT7-GUS transgenic plants, belonging to all lines, expressed T7-pol, as indicated by Western-blot analyses. Seedlings of these plants (2,100, belonging to 21 lines) were tested for GUS expression histochemically, or by assaying GUS activity in plant extracts.

T7-driven GUS activity could not be found in these plants. Lack of GUS expression was observed in the double-transformed plants regardless of the level of expression of T7-pol (Table 1). Only in two plants GUS staining was observed in pollen grains (Figure 2) and callus (data not shown). GUS activity was, however, fully silenced in the leaves of these 2 plants which was corroborated also by nuclear run-on and RNase protection assays.

Table 1 - GUS enzymatic activity

The transgenic plant	No. of tested plants	GUS activity (average) nmole 4-MU/h/mg protein
Non-transformed (SR1)	5	0.7
35S-GUS	5	272±71
T7-GUS	5	0.8
35S-T7-pol	5	0.6
35S-T7-pol/T7-GUS[a]	5	0.7
35S-T7-pol/T7-GUS/35S-GUS[b]	3	0.6

[a] double-transformed plants
[b] triple-transformed plants

Example 4 - Silencing of expressing a target gene via grafting

Transgenic RO tobacco plants carrying the p35S-RNA-pol construct were crossed with similar plants carrying the pT7-GUS construct (Fig. 1). The hybrid plants were self

pollinated and progeny were selected for several generations. At all stages plants were PCR-analyzed for both T7-pol and GUS, and only plants carrying the both genes were selected. Then, a scion from an expressing 35S_GUS plant was grafted upon the double-transformed, GUS-silenced plants. Shoots growing from the grafted scions were examined for GUS expression histochemically. Three out of the 6 plants in this experiment were silenced for GUS. Hence, in some cases, once the T7-derived silencing machinery was set in motion, it was capable of signal silencing to an already active pertinent gene across a graft.

Example 5 - Conferring plants with resistance to a plant pathogen (TMV)

Resistance to TMV in plants carrying the expression silencing system of the invention which included a TMV originated sequence (Ω) downstream to pT7 was also examined. Accordingly, several of the double-transformed plants were inoculated with TMV (10μg/ml in 0.01M phosphate buffer). Seven days post-inoculation, five leaf discs (6 mm in diameter) were cut of randomly from each plant and identically processed for ELISA with antibodies raised against the purified virus. The results presented in Table 1 show that all the double-transformed plant became partially or fully resistant to TMV (Table 2).

These results and the results presented for the silencing of GUS expression clearly indicate that the expression silencing system of the invention may carry a variety of targeting genes, homologous or heterologous, thereby silencing a broad spectrum of genes of interest.

Table 2 - ELISA readings for TMV in tobacco plants

Type of plant	Absorbance at 405 nm
Non-infected	0.038
Non-transgenic, TMV infected	0.614
Double transformant 1	0.208
Double transformant 2	0.022
Double transformant 3	0.250
Double transformant 4	0.087
Double transformant 5	0.015
Triple transformant 1	0.045
Triple transformant 2	0.008
Triple transformant 3	0.042

CLAIMS

1. An expression silencing system comprising

   a) a first DNA construct comprising a nucleotide sequence corresponding to the T7 RNA polymerase gene (T7-pol) or to a functional equivalent or fragment thereof which sequence carries an NLS sequence, and further comprising at least one promoter and at least one terminator sequence operably linked to said T7-pol; and

   b) a second DNA construct comprising a T7 promoter sequence (pT7) or a functional fragment thereof, at least one targeting sequence downstream to said pT7 and at least one 3` non-translated terminator sequence operably linked to said targeting sequence;

   which system is capable, upon introduction thereof into a cell, of rendering the expression at the RNA level of a target sequence in said cell, in a tissue or organ regenerated from said cell or in a progeny thereof, substantially silenced, by causing the substantial disappearance of the RNA or RNA transcript carrying said sequence or a functional part thereof.

2. A protein expression silencing system comprising a nucleotide sequence corresponding to the T7 RNA polymerase gene (T7-pol) or a functional equivalent or fragment thereof which sequence carries an NLS sequence, which construct further comprises at least one promoter and at least one terminator sequence operably linked to said T7-pol, a T7 promoter (pT7) or a functional equivalent or fragment thereof, at least one targeting sequence downstream to said the pT7, and at least one additional terminator sequence operably linked to said targeting sequence, said system being capable, upon introduction thereof into a cell, of rendering the expression at the RNA level of a target sequence in said cell, in a tissue or organ regenerated from said cell or in a progeny thereof, substantially silenced, by causing the substantial disappearance of the RNA or RNA transcript carrying said sequence.

3. The expression silencing system as claimed in claim 1 or claim 2, wherein said cell, in which the expression at the RNA level is substantially silenced is an eukaryotic

cell or a prokaryotic cell selected from a plant cell, a mammalian cell, a bacterium, a yeast, their pathogens, or any suitable tissue culture cell.

4. The expression-silencing system as claimed in claim 1 or claim 2, wherein said regenerated organ is a flowering differentiated plant regenerated from said cell.

5. The expression silencing system as claimed in claim 1 or claim 2, wherein said at least one targeting sequence substantially identical or homologous to at least part of said target sequence.

6. The expression silencing system as claimed in claim 5, wherein said target sequence corresponds to:-

   a) a gene encoding a protein or a peptide product, the silencing of which is desired;
   b) a non-coding nucleic acid sequence, which, under normal conditions, promotes the expression of an essential coding sequence;
   c) a nucleic acid sequence which corresponds to (a) or to (b) or to a fragment thereof, within the scope of degeneracy of the genetic code; or
   d) a nucleic acid sequence which hybridizes with the sequence according to (a), to (b), or to (c) or with fragments thereof, which hybridization is carried out under conditions which allow such hybridization to occur.

7. The expression silencing system as claimed in claim 6, wherein said gene encodes a plant protein or peptide product or a protein or peptide product of a plant pathogen.

8. The expression silencing system as claimed in claim 7, wherein said protein or peptide product of a plant pathogen is plant virus, a bacterium or a fungus capable of infecting said plant.

9. The expression silencing system as claimed in claim 7, wherein said gene encodes the GUS protein.

10. The expression silencing system as claimed in claim 6, wherein said gene encodes a human protein or peptide product or a protein or peptide product of a human pathogen.

11. The expression silencing system as claimed in claim 6, wherein said non-coding sequence is a regulatory element sequence which, under normal conditions, promotes the expression of a coding sequence.

12. The expression silencing system as claimed in claim 10, wherein said target sequence is the TMV non-coding sequence Ω.

13. The expression silencing system as claimed in claim 5, optionally further comprising additional regulatory elements.

14. The expression silencing system as claimed in claim 5, wherein said NLS sequence is the SV-40 NLS sequence.

15. The expression silencing system as claimed in claim 5, wherein said promoter sequence is the plant promoter p35S.

16. The expression silencing system as claimed in claim 1 or claim 2, wherein said terminator is the NOS terminator or a functional equivalent or fragment thereof, the β-1,3-gluconase terminator or any other suitable terminator capable of terminating the transcription of a nucleic acid sequence and of the addition of polyadenylated ribonucleotides to the 3` end of the primary transcript of said target sequence.

17. The expression silencing system as claimed in claim 1 or claim 2, wherein said pT7 corresponds to the promoter region of the bacteriophage T7 or functional analogues thereof, which promoter is capable of initiating transcription of said at least one targeting sequence downstream thereto.

18. The expression silencing system as claimed in claim 1 or claim 2, comprising the T7 terminator and the NOS terminator operably linked to said targeting sequence.

19. The expression silencing system as claimed in claim 1, wherein said first and second DNA constructs are substantially as shown in Figures 1A and 1B, respectively.

20. A process for the transformation of a plant with a gene-silencing system which process comprises:-

    a) transforming plant cells with:-

i) a first DNA construct comprising a nucleotide sequence corresponding to the T7 RNA polymerase gene (T7-pol) or a functional equivalent or fragment thereof, at least one plant promoter and at least one plant terminator sequence operably linked to said T7-pol;

and with

ii) a second DNA construct comprising a T7 promoter sequence or a functional fragment thereof, a targeting sequence downstream to said T7 promoter, and at least one 3` non-translated terminator sequence operably linked to said targeting sequence, said construct optionally further comprising other additional regulatory elements operably linked to said targeting sequence;

b) selecting the plant cells transformed with at least one DNA construct according to (a) and regenerating said selected cells to provide a differentiated flowering plant; and

c) hybridizing a plant transformed with said first DNA construct with a plant transformed with said second DNA construct, which first plant and second plant are obtained in (b), said hybridization thus providing a double-transformed plant in which the expression of a target sequence is substantially suppressed.

21. A process for the transformation of plant with a gene-silencing system, which process comprises:-

a) transforming plant cells with a DNA construct comprising a nucleotide sequence corresponding to the T7 RNA polymerase gene (T7-pol) or a functional equivalent or fragment thereof which sequence carries an NLS sequence, said construct further comprising at least one plant promoter sequence and at least one plant terminator sequence operably linked to said polymerase gene, a T7 promoter sequence (pT7) or a functional fragment thereof, a targeting sequence downstream to said pT7, and at least one additional terminator sequence operably linked to said targeting sequence, which DNA construct is capable, upon transformation thereof into a plant, of rendering the expression of a target sequence in said plant or in its progeny, substantially silenced; and

b) selecting plant cells transformed with said DNA construct according to (a) and regenerating said selected cells to provide a differentiated flowering plant.

22. The process as claimed in claim 20 or claim 21, wherein said targeting sequence substantially corresponds to said target sequence or to a fragment thereof.

23. The process as claimed in claim 22, wherein said target sequence corresponds to:-

a) a gene encoding a protein or a peptide product, the silencing of which is desired;

b) a non-coding nucleic acid sequence, which, under normal conditions, promotes the expression of an essential coding sequence;

c) a nucleic acid sequence which corresponds to (a) or to (b) or to a fragment thereof, within the scope of degeneracy of the genetic code; or

d) a nucleic acid sequence which hybridizes with the sequence according to (a), to (b), or to (c) or with fragments thereof, which hybridization is carried out under conditions which allow such hybridization to occur.

24. A method for producing a transgenic plant carrying a substantially silent target sequence, by hybridizing a plant carrying and expressing said target sequence with a transformed plant obtained by the process of claim 22.

25. A method for producing a transgenic plant carrying a substantially silent target sequence, by grafting a plant, or parts thereof, carrying and expressing said silent target sequence on a transformed plant obtained by the process of claim 22.

26. A transgenic plant or its progeny obtained by the method of claim 24 or claim 25, in which the expression of said target sequence is substantially suppressed.

27. A method of silencing the expression of a target sequence within the genome of a plant or within the genome of a plant infecting pathogen present in said cell prior to the following manipulation, which method comprises the steps of:

a) providing a first plant capable of regenerating;

b) hybridizing said first plant with a second plant double transformed with:-

i) a first DNA construct comprising a nucleotide sequence corresponding to the T7 RNA polymerase gene (T7-pol) or a functional equivalent or fragment thereof which sequence carries an NLS sequence, said construct

further comprising at least one plant promoter and at least one plant terminator sequence operably lined to said sequence;

and with

ii) a second DNA construct comprising a T7 promoter sequence (pT7), a targeting sequence downstream to said pT7 and a 3` non-translated terminator sequence operably linked to said targeting sequence, said construct optionally further comprising additional regulatory elements operably linked to said targeting sequence; and

c) selecting those plants obtained by the hybridization of step (b), in which the expression of said target sequence is substantially silenced.

28. A method of silencing the expression of a target sequence within the genome of a plant or within the genome of a plant infecting pathogen present in said cell prior to the following manipulation, which method comprises the steps of:-

a) providing a first plant comprising said target sequence, said plant being capable of regenerating;

b) hybridizing said first plant with a second plant transformed with a DNA construct comprising a nucleotide sequence corresponding to the T7 RNA polymerase gene (T7-pol) or a functional equivalent or fragment thereof which sequence carries an NLS sequence, said construct further comprising a plant promoter and a plant terminator sequence operably linked to said T7-pol, a T7 promoter (pT7) or a functional fragment thereof, a targeting sequence downstream to said pT7, and at least one additional promoter sequence operably linked to said targeting sequence; and

c) selecting those plants obtained by the hybridization of step (b), in which the expression of said target sequence is substantially silenced.

29. A method as claimed in claim 27 or claim 28, wherein said targeting sequence substantially corresponds said target sequence or to a fragment thereof.

30. The method as claimed in claim 28, wherein said target sequence corresponds to:-

a) a gene encoding a protein or a peptide product, the silencing of which is desired;

b) a non-coding nucleic acid sequence, which, under normal conditions, promotes the expression of an essential coding sequence;

c) a nucleic acid sequence which corresponds to (a) or to (b) or to a fragment thereof, within the scope of degeneracy of the genetic code; or

d) a nucleic acid sequence which hybridizes with the sequence according to (a), to (b), or to (c) or with fragments thereof, which hybridization is carried out under conditions which allow such hybridization to occur.

31. A method of identifying a nucleic acid of interest within a plant's genome wherein said nucleic acid of interest encodes a pre-defined plant phenotype, which process comprises the steps of:-

a) providing a first plant comprising within its genome said nucleic acid of interest;

b) transforming said first plant with a second plant transformed with:-

i) a first DNA construct comprising a nucleotide sequence corresponding to the T7 RNA polymerase gene (T7-pol) or a functional equivalent or fragment thereof which sequence carries an NLS sequence, said construct further comprising at least one plant promoter and at least one plant terminator sequence operably lined to said sequence;

and with

ii) a second DNA construct comprising a T7 promoter sequence, a random nucleic acid sequence downstream to said T7 promoter, and a 3` non-translated terminator sequence operably linked to said random nucleic acid sequence, said construct optionally further comprising additional regulatory elements operably linked to said nucleic acid of interest, said transformation thus provides a population of transgenic plants;

c) selecting from the population obtained in step (a) those transformed plants/plant cells in which the pre-defined phenotype is substantially silenced; and

d) employing said random nucleic acid sequence within the genome of transformed plants selected in step (c) as a probe in screening genomic DNA and cDNA libraries of said first plant, thereby identifying the gene comprising said random nucleic acid sequence which gene is responsible for said pre-defined phenotype.

32. A method of identifying a nucleic acid of interest within a plant's genome wherein said nucleic acid of interest encodes a pre-defined plant phenotype, which process comprises the steps of:-

a) providing a first plant comprising within its genome said nucleic acid of interest;

b) transforming said first plant with a second plant transformed with a DNA construct comprising a nucleotide sequence corresponding to the T7 RNA polymerase gene (T7-pol) or a functional equivalent or fragment thereof which sequence carries an NLS sequence, said construct further comprising at least one plant promoter sequence and at least one plant terminator sequence operably linked to said T7-pol, said DNA construct further comprising a T7 promoter sequence or a functional fragment thereof, a random nucleic acid sequence downstream to said T7 promoter, and a 3` non-translated terminator sequence operably linked to said random nucleic acid sequence;

c) selecting from the plants obtained in step (b) transformed plants in which the pre-defined phenotype is substantially silenced; and

d) employing said random nucleic acid sequence within the genome of the transformed plants selected in step (c) as a probe in screening genomic DNA or cDNA libraries of said first plant, thereby identifying the gene comprising said random nucleic acid sequence, which gene is responsible for said pre-defined phenotype.

Fig. 1A

Fig. 1B

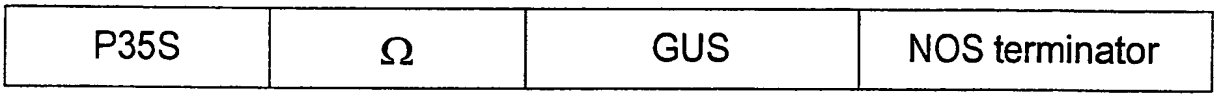

Fig. 1C

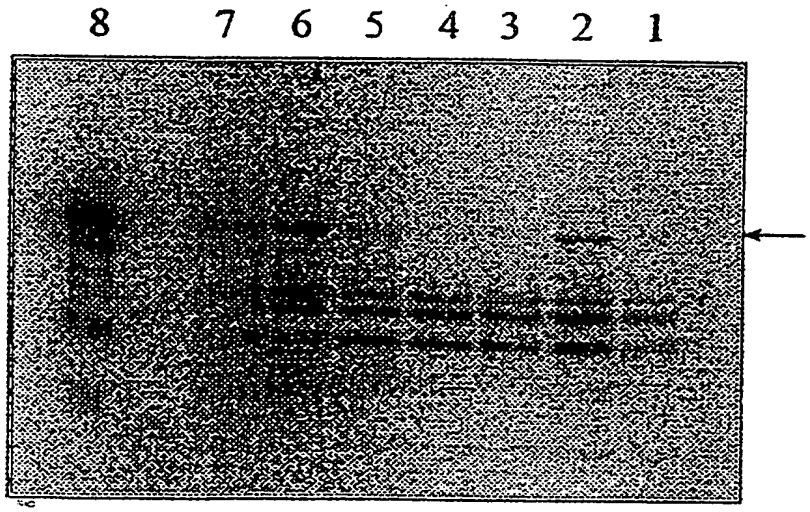

Fig. 2

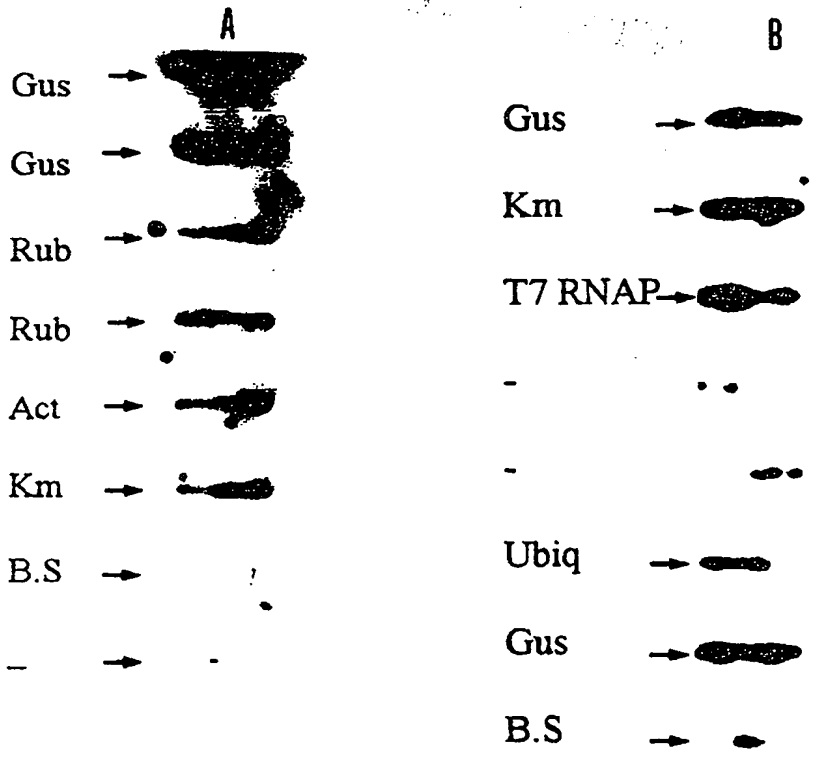

Fig. 3

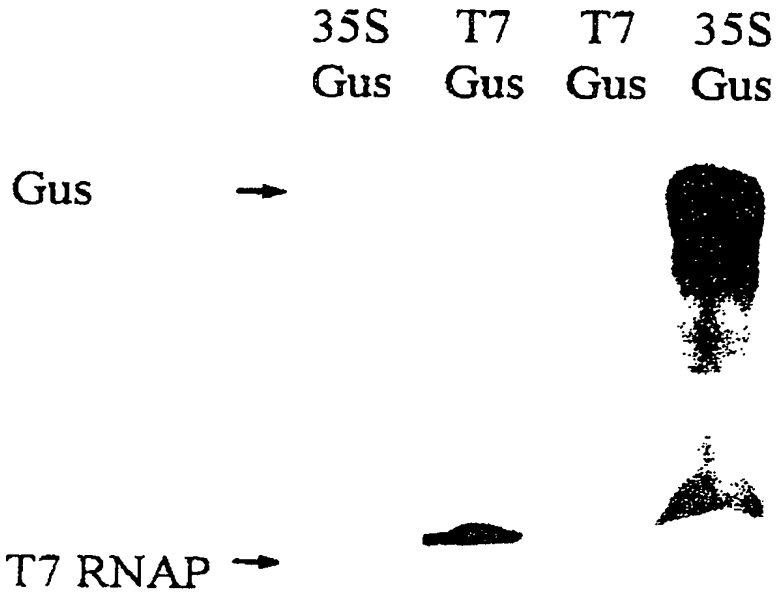

Fig. 4A

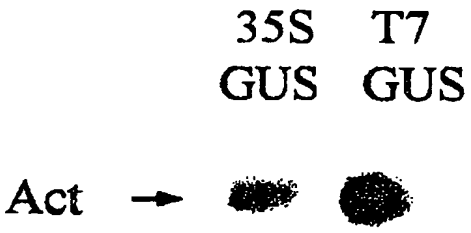

Fig. 4B

RECTIFIED BY
RULE 91 ISA/EP

11. The expression silencing system as claimed in claim 6, wherein said non-coding sequence is a regulatory element sequence which, under normal conditions, promotes the expression of a coding sequence.

12. The expression silencing system as claimed in claim 10, wherein said target sequence is the TMV non-coding sequence Ω.

13. The expression silencing system as claimed in claim 5, optionally further comprising additional regulatory elements.

14. The expression silencing system as claimed in claim 5, wherein said NLS sequence is the SV-40 NLS sequence.

15. The expression silencing system as claimed in claim 5, wherein said promoter sequence is the plant promoter p35S.

16. The expression silencing system as claimed in claim 1 or claim 2, wherein said terminator is the NOS terminator or a functional equivalent or fragment thereof, the β-1,3-gluconase terminator or any other suitable terminator capable of terminating the transcription of a nucleic acid sequence and of the addition of polyadenylated ribonucleotides to the 3' end of the primary transcript of said target sequence.

17. The expression silencing system as claimed in claim 1 or claim 2, wherein said pT7 corresponds to the promoter region of the bacteriophage T7 or functional analogues thereof, which promoter is capable of initiating transcription of said at least one targeting sequence downstream thereto.

18. The expression silencing system as claimed in claim 1 or claim 2, comprising the T7 terminator and the NOS terminator operably linked to said targeting sequence.

19. The expression silencing system as claimed in claim 1, wherein said first and second DNA constructs are substantially as shown in Figures 1A and 1B, respectively.

20. A process for the transformation of a plant with a gene-silencing system which process comprises:-

a) transforming plant cells with:-

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 14 September 2000 (14.09.00)	
International application No. PCT/IL00/00029	**Applicant's or agent's file reference** 6039/WO/99
International filing date (day/month/year) 16 January 2000 (16.01.00)	**Priority date** (day/month/year) 18 January 1999 (18.01.99)
Applicant SELA, Ilan et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

03 August 2000 (03.08.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Charlotte ENGER
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
6039/WO/99		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/IL 00/ 00029	16/01/2000	18/01/1999
Applicant		
YISSUM RESEARCH DEVELOPMENT COMPANY .et al		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. 1

   [ ] as suggested by the applicant.

   [X] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [ ] None of the figures.

INTERNATIONAL SEARCH REPORT

Inte[redacted]pplication No
PCT/IL 00/00029

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/82 C12N15/63 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	LASSNER, M.W. ET AL.: "Targeting of T7 RNA polymerase to tobacco nuclei mediated by an SV40 nuclear localization signal." PLANT MOLECULAR BIOLOGY, vol. 17, 1991, pages 229-34, XP002139111 cited in the application the whole document	1-32
Y	WO 98 36083 A (ANGELL SUSAN MARY ;BAULCOMBE DAVID CHARLES (GB); PLANT BIOSCIENCE) 20 August 1998 (1998-08-20) the whole document	1-32
A	WO 98 34951 A (STANLEY EDOUARD G ;FABRI LOUIS (AU); LAH MARIA (AU); NASH ANDREW D) 13 August 1998 (1998-08-13) page 26, line 11 - line 29	10

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 May 2000	20/06/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Smalt, R

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	WO 98 53083 A (LOWE ALEXANDRA LOUISE ;GRIERSON DONALD (GB); ZENECA LTD (GB); HAMI) 26 November 1998 (1998-11-26) the whole document	11
A	WO 97 27295 A (MANNING KENNETH ;HORTICULTURE RES INT (GB)) 31 July 1997 (1997-07-31) page 3, line 2 - line 5	31,32
A	MONTGOMERY M K ET AL: "Double-stranded RNA as a mediator in sequence-specific genetic silencing and co-suppression" TRENDS IN GENETICS,NL,ELSEVIER SCIENCE PUBLISHERS B.V. AMSTERDAM, vol. 14, no. 7, 1 July 1998 (1998-07-01), pages 255-258, XP004124680 ISSN: 0168-9525 figure 1D	
P,X	ZEITOUNE, S. ET AL.: "T7 RNA polymerase drives transcription of a reporter gene from T7 promoter, but engenders post-transcriptional silencing of expression." PLANT SCIENCE, vol. 141, no. 1, 2 February 1999 (1999-02-02), pages 59-65, XP000913733 the whole document	1-32

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. Application No
PCT/IL 00/00029

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9836083	A	20-08-1998	AU	6001698 A	08-09-1998
			EP	0970228 A	12-01-2000
WO 9834951	A	13-08-1998	AU	5848698 A	26-08-1998
WO 9853083	A	26-11-1998	AU	7444298 A	11-12-1998
			EP	0983370 A	08-03-2000
WO 9727295	A	31-07-1997	AU	1316297 A	20-08-1997
			EP	0876481 A	11-11-1998

OIPE JC81 PATENT OFFICE JUL 18 2001

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
0039/WO/99		
International application No.	International filing date (day/month/year)	Priority date (day/month/year)
PCT/IL00/00029	16/01/2000	18/01/1999

International Patent Classification (IPC) or national classification and IPC
C12N15/82

Applicant
YISSUM RESEARCH DEVELOPMENT COMPANY .et al

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 8 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 1 sheets.

3. This report contains indications relating to the following items:

   I ☒ Basis of the report
   II ☐ Priority
   III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   IV ☐ Lack of unity of invention
   V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   VI ☐ Certain documents cited
   VII ☒ Certain defects in the international application .
   VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
03/08/2000	01.06.2001

Name and mailing address of the international preliminary examining authority:
European Patent Office
D-80298 Munich
Tel. +49 89 2399 - 0 Tx: 523656 epmu d
Fax: +49 89 2399 - 4465

Authorized officer
Wimmer, G
Telephone No. +49 89 2399 7347

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/IL00/00029

I. Basis of the report

1. With regard to the elements of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-22	as originally filed

Claims, No.:

1-11,13-15, 18-32	as originally filed	
12,16,17	with telefax of	06/03/2001

Drawings, sheets:

1/3-3/3	as originally filed

2. With regard to the language, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rul 55.2 and/or 55.3).

3. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

INTERNATIONAL PRELIMINARY EXAMINATION REPORT - SEPARATE SHEET

International application No. PCT/IL00/00029

Re Item V

Reasoned statement under Art. 35(2) PCT with regard to novelty, inventive step or industrial applicability.

1) Reference is made to the following documents (the document numbering corresponds to their order of citation in the international search report):

D1: LASSNER, M.W. ET AL.: 'Targeting of T7 RNA polymerase to tobacco nuclei mediated by an SV40 nuclear localization signal.' PLANT MOLECULAR BIOLOGY, vol. 17, 1991, pages 229-34, XP002139111 cited in the application

D2: WO 98 36083 A (ANGELL SUSAN MARY ;BAULCOMBE DAVID CHARLES (GB); PLANT BIOSCIENCE) 20 August 1998 (1998-08-20)

D6: MONTGOMERY M K ET AL: 'Double-stranded RNA as a mediator in sequence-specific genetic silencing and co-suppression' TRENDS IN GENETICS,NL,ELSEVIER SCIENCE PUBLISHERS B.V. AMSTERDAM, vol. 14, no. 7, 1 July 1998 (1998-07-01), pages 255-258, XP004124680 ISSN: 0168-9525

Priority as claimed appears to be valid. Document D7 as listed in the International Search Report (XP000913733) was therefore not regarded as prior art in this examination.

Novelty under Art. 33(2) PCT.

2) Although the formulations of claims 1 and 2 also embrace expression silencing through transcription of antisense RNA, in any organism, the prior art as made available by the international search does not disclose such a silencing system employing transcription through a T7 polymerase/promoter system having all the features of claims 1 or 2, respectively.
Claims 1-32 are therefore regarded as being novel.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/IL00/00029

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-32
	No:	Claims	
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-32
Industrial applicability (IA)	Yes:	Claims	1-32
	No:	Claims	

2. Citations and explanations
see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

Inventive Step under Art. 33(3) PCT.

3) The invention of the present application is a gene silencing system, in which transcription of the targeting sequence is driven by T7 polymerase.

Document D2 describes a system, preferably for use in plants, by which expression of specific proteins can be silenced. The method of D2 describes ways to achieve this through the employment of antisense inhibition or of the cosuppression phenomenon.
Specifically, D2 discloses constructs which include the bacterial GUS gene, and the NOS terminator sequence. Transcription of the targeting sequence is preferentially driven by endogenous Polymerase II and the plant S35 promoter.

The technical problem solved by the application was therefore the provision of an alternative polymerase/promoter system for the expression of the targeting sequence.

Document D1 describes the successful employment of T7 polymerase expression in a plant system. In this, the authors report that plant cells transfected with the T7 polymerase gene express functional polymerase. Moreover, the authors of D2 disclose that allocation of the polymerase to the nucleus is highly effective, through the use of SV40 Nuclear Localization Signal. The document also proposes this system for high-level expression if introduced genes in plant cells.

To solve the technical problem as formulated above, the skilled person would search the prior art for promoters which are useful for expression, preferentially high-level expression, in plant cells. Through this, the skilled person would employ the polymerase/promoter system of D1, in the silencing system of D2, and arrive at the method of the current application.

However, applicants argument that the skilled person would look for other plant promoters, rather than for exogenous promoters which have been shown functional in plants, since the applicants show that overexpression of the gene is not involved in the silencing mechanism, and was therefore not a desired function of the promoter. Therefore, applicants argue that the skilled person would not

have tried to employ the T7 promoter in gene silencing in plants.

The IPEA must disagree with this view. First, and as outlined in section VIII.2, the methods of the application as defined through the claims do not appear to be limited to cosuppression silencing in plants, but include also other (e.g. antisense silencing) methods in other organisms, e.g. mammals, for which cosuppression silencing has neither been shown in the prior art nor by the applicants. The use of a T7 polymerase/promoter system therein is thus not beyond routinely envisioned methods for the skilled person.

Moreover, the mechanism of cosuppression in plants was not fully understood at the time of the application, and therefore overexpression of a gene could not have been deemed detrimental to this effect - in fact, D2 proposes to use constitutively acting and highly expressing promoters in these methods, and the skilled person would therefore also have tried non-plant promoters, which have been shown to be active (and preferentially, highly active) in plants. The fact that the applicants found thereafter, that the capability of of high expression of the T7 polymerase/promoter system is not crucial for gene silencing, is irrelevant to the question of inventive step for the employment of this system.

Furthermore, it is evident from the description that it was the intention of the applicants to establish *over-expression* of a gene in plants by employing the T7 promoter/polymerase system, which, through the findings of D1, was already to be expected to function and which for the skilled person was therefore obvious to try. In this, the applicants found that T7-driven expression can lead to cosuppression in plants; this, in addition to the fact that (through the teachings of e.g. D2) such a result was at least not unlikely, can therefore be seen as a "bonus effect" of a method which for the skilled person was obvious to perform.

The IPEA must therefore decline to acknowledge the involvement of an inventive step for the methods of claims 1-30.

4) Systems for the identification of a specific gene, through introduction of random antisense sequences and isolation of a specific phenotype, are known in the art (see e.g. Kiecil et al. (1990), Mol.Med.Today 4(8): 268-74). By the same

reasoning as given in section V.3, the use of a T7 polymerase/promoter in such a system for plant cells, as referred to in claims 31 and 32, is lacking an inventive step. Consequently, also claims 31 and 32 do not comply with Art. 33(3) PCT.

Industrial Applicability under Art. 33(4) PCT.

5) Claim 10 is directed to possible treatment of human cells.
For the assessment of this claim on the question whether it is industrially applicable; no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known com- pound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

Re Item VII
Certain defects in the application.

1) Although formulated as a dependent claim, claim 17 does not fall within the terms of claims 1 and 2. The range of the feature "... the promoter sequence of the bacteriophage T7 *or functional analogues thereof* ..." is broader than that of "a T7 promoter sequence ... or a functional fragment thereof", since an analogue of the T7 promoter does not have sequence identity with the T7 promoter, and not necessarily with a functional fragment thereof.
This does not comply with the conciseness requirement of Art. 6 PCT; however, the claim was examined in the light that specificity for the T7 polymerase is required.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT - SEPARATE SHEET

International application No. PCT/IL00/00029

Re Item VIII

Certain observations and clarity.

1) The formulations "a functional equivalent or fragment thereof", "a functional fragment thereof" (claims 1 and 2) are not clear, as they are open to wide interpretation. For example, a functional fragment of a promoter could be limited to e.g. the TATA box.
Although the applicants refer to page 8, lines 1-5 of the description to clarify the term "functional equivalent" and "functional fragment", the terms remain unclear, since the definition therein only requires that such a promoter would allow for the expression of a functional target protein. Therefore, any promoter functional in plants, which contains even minor sequence identity with the T7 promoter (such as, containing a TATA box), would fall within these terms.
However, for the purpose of this examination, it was assumed that *specificity for the T7 polymerase* is required as a necessary function of the promoter sequence in question.

2) The applicability of the cosuppression method, on mammalian and especially human cells as included in claims 1 and 2, and specifically referred to in claims 3 and 10, is highly dubious and not supported by the description in a more than speculative manner (see Art. 6 PCT). Gene silencing through cosuppression has been described for certain organisms only - mainly for plants, but in special cases also for also nematodes (see document D6) or Drosophila. Thus, the application would not be enabling the skilled person to perform this cosuppression technique for *any* gene in *any* organism (claims 1-6), and especially not in human cells (claim 10), and would therefore not comply with Art. 5 PCT.
Applicants wished to defer dealing with this issue to the national phase.

Since anticense inhibition is also encompassed by the terms of the claims, all claims were examined for novelty, inventive step and industrial applicability.

11. The expression silencing system as claimed in claim 6, wherein said non-coding sequence is a regulatory element sequence which, under normal conditions, promotes the expression of a coding sequence.

12. The expression silencing system as claimed in claim 11, wherein said target sequence is the TMV non-coding sequence Ω.

13. The expression silencing system as claimed in claim 5, optionally further comprising additional regulatory elements.

14. The expression silencing system as claimed in claim 5, wherein said NLS sequence is the SV-40 NLS sequence.

15. The expression silencing system as claimed in claim 5, wherein said promoter sequence is the plant promoter p35S.

16. The expression silencing system as claimed in claim 1 or claim 2, wherein any or both of said terminators is the NOS terminator or a functional equivalent or fragment thereof, the β-1,3-gluconase terminator or any other suitable terminator capable of terminating the transcription of a nucleic acid sequence and of the addition of polyadenylated ribonucleotides to the 3' end of the primary transcript of said nucleic acid.

17. The expression silencing system as claimed in claim 1 or claim 2, wherein said pT7 corresponds to the promoter sequence of the bacteriophage T7 or functional analogues thereof, which promoter is capable of initiating transcription of said at least one targeting sequence downstream thereto.

18. The expression silencing system as claimed in claim 1 or claim 2, comprising the T7 terminator and the NOS terminator operably linked to said targeting sequence.

19. The expression silencing system as claimed in claim 1, wherein said first and second DNA constructs are substantially as shown in Figures 1A and 1B, respectively.

20. A process for the transformation of a plant with a gene-silencing system which process comprises:-

a) transforming plant cells with:-

OIPE JC87 JUL 18 2001 PATENT & TRADEMARK OFFICE

IPEA/ EP

PCT

CHAPTER II

DEMAND

under Article 31 of the Patent Cooperation Treaty:
The undersigned requests that the international application specified below be the subject of international preliminary examination according to the Patent Cooperation Treaty.

For International Preliminary Examining Authority use only

Identification of IPEA	Date of receipt of DEMAND

Box No. I IDENTIFICATION OF THE INTERNATIONAL APPLICATION		Applicant's or agent's file reference 6039/WO/99
International application No. PCT/IL00/00029	International filing date (day/month/year) 16 January 2000 (16.01.2000)	(Earliest) Priority date (day/month/year) 18 January 1999 (18.01.99)

Title of invention

AN EXPRESSION SILENCING SYSTEM AND DIFFERENT USES THEREOF

Box No. II APPLICANT(S)

Name and address:

YISSUM RESEARCH DEVELOPMENT COMPANY OF THE HEBREW UNIVERSITY OF JERUSALEM.
46 Jabotinsky Street
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Telephone No.:

Facsimile No.:

Teleprinter No.:

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State (i.e. country) of residence: IL

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Nes Ziona 74037
Israel

State (i.e. country) of nationality: IL

State (i.e. country) of residence: IL

Name and address:

ZEITOUNE-SIMOVICH, Sylvia
14 Cremieux Street
Tel Aviv 64259
Israel

State (i.e. country) of nationality: IL

State (i.e. country) of residence: IL

☐ Further applicants are indicated in a continuation sheet.

Inter.. ..onal application No.
PCT/IL00/00029

Box No. III AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The following person is ☒ agent ☐ common representative

and ☒ has been appointed earlier and represents the applicant(s) also for international preliminary examination.

☐ is hereby appointed and any earlier appointment of (an) agent(s)/common representative is hereby revoked.

☐ is hereby appointed, specifically for the procedure before the International Preliminary Examining Authority, in addition to the agent(s)/common representative appointed earlier.

Name and address:

LUZZATTO, Kfir; LUZZATTO, Edgar; LUZZATTO, Esther; HACKMEY, Michal; FUERST, Zadok; PYERNIK, Moshe; MANZUROLA, Emanuel; SERITYA, Yehuda; PRICE, Eyal; SHALEV, Ronit; HACKMEY, Miriam; DEN-HORIN, Hevion; RUTMAN, Avraham; CHECHIK, Haim
LUZZATTO & LUZZATTO
P.O.Box 5352
Beer-Sheva 84 152
Israel

Telephone No.:
(972-7) 6497-871

Facsimile No.:
(972-7) 6497-125

Teleprinter No.:

☐ Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent

Box No. IV STATEMENT CONCERNING AMENDMENTS

The applicant wishes the International Preliminary Examining Authority*

(i) ☒ to start the international preliminary examination on the basis of the international application as originally filed.

(ii) ☐ to take into account the amendments under Article 34 of

☐ the description (amendments attached).

☐ the claims (amendments attached).

☐ the drawings (amendments attached).

(iii) ☐ to take into account any amendments of the claims under Article 19 filed with the International Bureau (a copy is attached).

(iv) ☐ to disregard any amendments of the claims made under Article 19 and to consider them as reversed.

(v) ☐ to postpone the start of the international preliminary examination until the expiration of 20 months from the priority date unless that Authority receives a copy of any amendments made under Article 19 or a notice from the applicant that he does not wish to make such amendments (Rule 69.1(d)).

* Where no check-box is marked, international preliminary examination will start on the basis of the international application as originally filed or, where a copy of amendments to the claims under Article 19 and/or amendments to the international application under Article 34 are received by the International Preliminary Examining Authority before it has begun to draw up a written opinion or the international preliminary examination report, as so amended.

Box No. V ELECTION OF STATES

☒ The applicant hereby elects all eligible States except .....

...ernati nal application No.
PCT/IL00/00029

Box No. VI CHECK LIST

The demand is accompanied by the following documents for the purposes of international preliminary examination:

			For International Preliminary Examining Authority use only: received	not received
1. amendments under Article 34				
description	:	sheets	☐	☐
claims	:	sheets	☐	☐
drawings	:	sheets	☐	☐
2. letter accompanying amendments under Article 34	:	sheets	☐	☐
3. copy of amendments under Article 19	:	sheets	☐	☐
4. copy of statement under Article 19	:	sheets	☐	☐
5. other *(specify)*:	:	sheets	☐	☐

The demand is also accompanied by the item(s) marked below:

1. ☒ separate signed power of attorney
2. ☒ copy of general power of attorney
3. ☐ statement explaining lack of signature
4. ☒ fee calculation sheet
5. ☒ other *(specify)*: notification of bank transfer

Box No. VII SIGNATURE OF APPLICANT, AGENT OR COMMON REPRESENTATIVE

Michal Hackmey

For International Preliminary Examining Authority use only

1. Date of actual receipt of DEMAND:
2. Adjusted date of receipt of demand due to CORRECTIONS under Rule 60.1(b):
3. ☐ The date of receipt of the demand is AFTER the expiration of 19 months from the priority date and item 4 or 5, below, does not apply. ☐ The applicant has been informed accordingly.
4. ☐ The date of receipt of the demand is WITHIN the period of 19 months from the priority date as extended by virtue of Rule 80.5.
5. ☐ Although the date of receipt of the demand is after the expiration of 19 months from the priority date, the delay in arrival is EXCUSED pursuant to Rule 82.

For International Bureau use only

Demand received from IPEA on:

OIPE JC87 JUL 18 2001 PATENT & TRADEMARK OFFICE

PCT

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty

For receiving Office use only

International Application No.

International Filing Date

Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference *(if desired) (12 characters maximum)* 6039/WO/99

Box No. I TITLE OF INVENTION

AN EXPRESSION SILENCING SYSTEM AND DIFFERENT USES THEREOF

Box No. II APPLICANT

Name and address:

YISSUM RESEARCH DEVELOPMENT COMPANY OF THE HEBREW UNIVERSITY OF JERUSALEM
46 Jabotinsky Street
P.O. Box 4279
Jerusalem 91042
Israel

☐ This person is also an inventor.

Telephone No.

Facsimile No.

Teleprinter No.

State *(i.e. country)* of nationality: IL

State *(i.e. country)* of residence: IL

This person is applicant for the purposes of: ☐ all designated States ☒ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

Name and address:

SELA, Ilan
113 Ha'banim Street
Nes Ziona 74037
Israel

This person is:

☐ applicant only

☒ applicant and inventor

☐ inventor only

State *(i.e. country)* of nationality: IL

State *(i.e. country)* of residence: IL

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

☒ Further applicants and/or (further) inventors are indicated on a continuation sheet

Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☒ agent ☐ common representative

Name and address:

LUZZATTO, Kfir
LUZZATTO & LUZZATTO
P.O.Box 5352
Beer-Sheva 84 152
Israel

Telephone No.
(972-7) 6497-871

Facsimile No.
(972-7) 6497-125

Teleprinter No.

☐ Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Sheet No. ...2....

Continuation of Box No. III **FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)**

If none of the following sub-boxes is used, this sheet is not to be included in the request

Name and address:

ZEITOUNE-SIMOVICH, Sylvia
14 Cremieux Street
Tel Aviv 64259
Israel

This person is:
- [ ] applicant only
- [x] applicant and inventor
- [ ] inventor only

State (*i.e. country*) of nationality: IL | State (*i.e. country*) of residence: IL

This person is applicant for the purposes of: [ ] all designated States [ ] all designated States except the United States of America [x] the United States of America only [ ] the States indicated in the Supplemental Box

Name and address:

This person is:
- [ ] applicant only
- [ ] applicant and inventor
- [ ] inventor only

State (*i.e. country*) of nationality: | State (*i.e. country*) of residence:

This person is applicant for the purposes of: [ ] all designated States [ ] all designated States except the United States of America [ ] the United States of America only [ ] the States indicated in the Supplemental Box

Name and address:

This person is:
- [ ] applicant only
- [ ] applicant and inventor
- [ ] inventor only

State (*i.e. country*) of nationality: | State (*i.e. country*) of residence:

This person is applicant for the purposes of: [ ] all designated States [ ] all designated States except the United States of America [ ] the United States of America only [ ] the States indicated in the Supplemental Box

Name and address:

This person is:
- [ ] applicant only
- [ ] applicant and inventor
- [ ] inventor only

State (*i.e. country*) of nationality: | State (*i.e. country*) of residence:

This person is applicant for the purposes of: [ ] all designated States [ ] all designated States except the United States of America [ ] the United States of America only [ ] the States indicated in the Supplemental Box

[ ] Further applicants and/or (further) inventors are indicated on a continuation sheet

Box No. V DESIGNATION OF STATES

The following designations are hereby made under Rule 4.9(a) *(mark the applicable check-boxes; at least one must be marked)*:

Regional Patent

- ☒ **AP** ARIPO Patent: GH Ghana, GM Gambia, KE Kenya, LS Lesotho, MW Malawi, SD Sudan, SL Sierra Leone, SZ Swaziland, TZ United Republic of Tanzania, UG Uganda, ZW Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT
- ☒ **EA** Eurasian Patent: AM Armenia, AZ Azerbaijan, BY Belarus, KG Kyrgyzstan, KZ Kazakhstan, MD Republic of Moldova, RU Russian Federation, TJ Tajikistan, TM Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT
- ☒ **EP** European Patent: AT Austria, BE Belgium, CH and LI Switzerland and Liechtenstein, CY Cyprus, DE Germany, DK Denmark, ES Spain, FI Finland, FR France, GB United Kingdom, GR Greece, IE Ireland, IT Italy, LU Luxembourg, MC Monaco, NL Netherlands, PT Portugal, SE Sweden, and any other State which is a Contracting State of the European Patent Convention and of the PCT
- ☒ **OA** OAPI Patent: BF Burkina Faso, BJ Benin, CF Central African Republic, CG Congo, CI Côte d'Ivoire, CM Cameroon, GA Gabon, GN Guinea, GW Guinea-Bissau, ML Mali, MR Mauritania, NE Niger, SN Senegal, TD Chad, TG Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT *(if other kind of protection or treatment desired, specify on dotted line)* .....

National Patent *(if other kind of protection or treatment desired, specify on dotted line)*:

- ☒ AE United Arab Emirates
- ☒ AL Albania
- ☒ AM Armenia
- ☒ AT Austria
- ☒ AU Australia
- ☒ AZ Azerbaijan
- ☒ BA Bosnia and Herzegovina
- ☒ BB Barbados
- ☒ BG Bulgaria
- ☒ BR Brazil
- ☒ BY Belarus
- ☒ CA Canada
- ☒ CH and LI Switzerland and Liechtenstein
- ☒ CN China
- ☒ CR Costa Rica
- ☒ CU Cuba
- ☒ CZ Czech Republic
- ☒ DE Germany
- ☒ DK Denmark
- ☒ DM Dominica
- ☒ EE Estonia
- ☒ ES Spain
- ☒ FI Finland
- ☒ GB United Kingdom
- ☒ GD Grenada
- ☒ GE Georgia
- ☒ GH Ghana
- ☒ GM Gambia
- ☒ HR Croatia
- ☒ HU Hungary
- ☒ ID Indonesia
- ☒ IL Israel
- ☒ IN India
- ☒ IS Iceland
- ☒ JP Japan
- ☒ KE Kenya
- ☒ KG Kyrgyzstan
- ☒ KP Democratic People's Republic of Korea
- ☒ KR Republic of Korea
- ☒ KZ Kazakhstan
- ☒ LC Saint Lucia
- ☒ LK Sri Lanka
- ☒ LR Liberia
- ☒ LS Lesotho
- ☒ LT Lithuania
- ☒ LU Luxembourg
- ☒ LV Latvia
- ☒ MA Morocco
- ☒ MD Republic of Moldova
- ☒ MG Madagascar
- ☒ MK The former Yugoslav Republic of Macedonia
- ☒ MN Mongolia
- ☒ MW Malawi
- ☒ MX Mexico
- ☒ NO Norway
- ☒ NZ New Zealand
- ☒ PL Poland
- ☒ PT Portugal
- ☒ RO Romania
- ☒ RU Russian Federation
- ☒ SD Sudan
- ☒ SE Sweden
- ☒ SG Singapore
- ☒ SI Slovenia
- ☒ SK Slovakia
- ☒ SL Sierra Leone
- ☒ TJ Tajikistan
- ☒ TM Turkmenistan
- ☒ TR Turkey
- ☒ TT Trinidad and Tobago
- ☒ TZ United Republic of Tanzania
- ☒ UA Ukraine
- ☒ UG Uganda
- ☒ US United States of America
- ☒ UZ Uzbekistan
- ☒ VN Viet Nam
- ☒ YU Yugoslavia
- ☒ ZA South Africa
- ☒ ZW Zimbabwe

Check-boxes reserved for designating States which have become party to the PCT after issuance of this sheet:

- ☐ .....
- ☐ .....

Precautionary Designation Statement: In addition to the designations made above, the applicant also makes under Rule 4.9(b) all other designations which would be permitted under the PCT except any designation(s) indicated in the Supplemental Box as being excluded from the scope of this statement. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. *(Confirmation (including fees) must reach the receiving Office within the 15-month time limit.)*

Supplemental Box — *If the supplemental Box is not used, this sheet need not be included in the request.*

Continuation of Box No. IV

LUZZATTO, Edgar
LUZZATTO, Esther
HACKMEY, Michal
FUERST, Zadok
PYERNIK, Moshe
MANZUROLA, Emanuel
SERUYA, Yehuda
CHECHIK, Haim
PRICE, Eyal
SHALEV, Ronit
HACKMEY, Miriam

P.O.Box 5352
Beer-Sheva 84 152
Israel

Box No. VI PRIORITY CLAIM Further priority claims are indicated in the Supplemental Box ☐

The priority of the following earlier application(s) is hereby claimed:

Country *(in which, or for which the application was filed)*	Filing Date *(day/month/year)*	Application No.	Office of filing *(only for regional or international application)*
item(1) IL	18 January 1999 (18.01.99)	128111	
item(2)			
item(3)			

Mark the following check-box if the certified copy of the earlier application is to be issued by the Office which for the purposes of the present international application is the receiving Office (a fee may be required):

☒ The receiving Office is hereby requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) identified above as item(s): 1

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) *(If two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used):* ISA / EP

Earlier Search *Fill in where a search (international, international-type or other) by the International Searching Authority has already been carried out or requested and the Authority is now requested to base the international search, to the extent possible, on the results of that earlier search. Identify such search or request either by reference to the relevant application (or the translation thereof) or by reference to the search request:*

Country (or regional Office): Date *(day/month/year)*: Number:

Box No. VIII CHECK LIST

This international application contains the following number of sheets:

1. request	:	5 sheets
2. description	:	22 sheets
3. claims	:	8 sheets
4. abstract	:	1 sheet
5. drawings:		3 sheets
Total	:	39 sheets

This international application is accompanied

1. ☐ separate signed power of attorney
2. ☒ copy of general power of attorney
3. ☐ statement explaining lack of signature
4. ☐ priority document(s) *identified in Box No. VI as item(s):*
5. ☒ fee calculation sheet
6. ☐ separate indications concerning deposited microorganisms
7. ☐ nucleotide and/or amino acid sequence listing (diskette)
8. ☐ other *(specify)*

Figure No. of the drawings (if any) should accompany the abstract when it is published.

Box No. IX SIGNATURE OF APPLICANT OR AGENT

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request)

Kfir Luzzatto

For receiving Office use only

1. Date of actual receipt of the purported international application: 518 Rec'd PCT/PTO 18 JUL 2001
2. Drawings: ☐ received: ☐ not received:
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:
4. Date of timely receipt of the required corrections under PCT Article 11(2):
5. International Searching Authority specified by the applicant: ISA/
6. ☐ Transmittal of search copy delayed until search fee is paid

For International Bureau use only

Form PCT/RO/101 (last sheet) (January 1994)

Date of receipt of the record copy by the International Bureau:

OIPE JC81 JUL 18 2001 PATENT & TRADEMARK OFFICE

WO 00/42206
PCT/IL00/0002

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

To:
LUZZATTO, Kfir
Luzzatto & Luzzatto
P.O. Box 5352
84152 Beer-Sheva
ISRAEL

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

Date of mailing (day/month/year)	
20 July 2000 (20.07.00)	
Applicant's or agent's file reference	**IMPORTANT NOTICE**
6039/WO/99	

International application No.	International filing date (day/month/year)	Priority date (day/month/year)
PCT/IL00/00029	16 January 2000 (16.01.00)	18 January 1999 (18.01.99)

Applicant: YISSUM RESEARCH DEVELOPMENT COMPANY OF THE HEBREW UNIVERSITY OF JERUSALEM et al

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this Notice:
   AU,CN,JP,KP,KR,US

   In accordance with Rule 47.1(c), third sentence, those Offices will accept the present Notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:
   AE,AL,AM,AP,AT,AZ,BA,BB,BG,BR,BY,CA,CH,CR,CU,CZ,DE,DK,DM,EA,EE,EP,ES,FI,GB,GD,GE,GH,GM,HR,HU,ID,IL,IN,IS,KE,KG,KZ,LC,LK,LR,LS,LT,LU,LV,MA,MD,MG,MK,MN,MW,MX,NO,NZ,OA,PL,PT,RO,RU,SD,SE,SG,SI,SK,SL,TJ,TM,TR,TT,TZ,UA,UG,UZ,VN,YU,ZA,ZW
   The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49.1(a-bis)).

3. Enclosed with this Notice is a copy of the international application as published by the International Bureau on
   20 July 2000 (20.07.00) under No. WO 00/42200

REMINDER REGARDING CHAPTER II (Article 31(2)(a) and Rule 54.2)

If the applicant wishes to postpone entry into the national phase until 30 months (or later in some Offices) from the priority date, a demand for international preliminary examination must be filed with the competent International Preliminary Examining Authority before the expiration of 19 months from the priority date.

It is the applicant's sole responsibility to monitor the 19-month time limit.

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

REMINDER REGARDING ENTRY INTO THE NATIONAL PHASE (Article 22 or 39(1))

If the applicant wishes to proceed with the international application in the national phase, he must, within 20 months or 30 months, or later in some Offices, perform the acts referred to therein before each designated or elected Office.

For further important information on the time limits and acts to be performed for entering the national phase, see the Annex to Form PCT/IB/301 (Notification of Receipt of Record Copy) and Volume II of the PCT Applicant's Guide.

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Facsimile No. (41-22) 740.14.35

Authorized officer
J. Zahra
Telephone No. (41-22) 338.83.38

Continuation 1 Form PCT/IB/308

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

Date of mailing (day/month/year) 20 July 2000 (20.07.00)	IMPORTANT NOTICE
Applicant's or agent's file reference 6039/WO/99	International application No. PCT/IL00/00029

The applicant is hereby notified that, at the time of establishment of this Notice, the time limit under Rule 46.1 for making amendments under Article 19 has not yet expired and the International Bureau had received neither such amendments nor a declaration that the applicant does not wish to make amendments.